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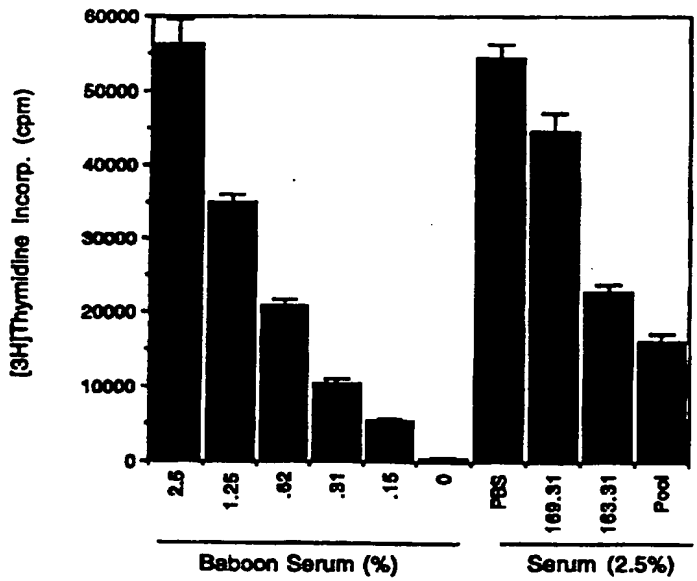
(54) Title: COMPOSITION FOR INHIBITING INTIMAL HYPERPLASIA USING PDGF ANTAGONISTS AND HEPARIN



(57) Abstract

Methods for inhibiting intimal hyperplasia in the vasculature of mammals, including primates, are disclosed. The methods comprise coordinately administering to the mammal a PDGF antagonist and heparin. The antagonist can be a non-peptidic antagonist or an anti-PDGF receptor antibody, such as an anti-PDGF-alpha receptor antibody or an anti-PDGF-beta receptor antibody. The methods are useful in reducing intimal hyperplasia due to, for example, vascular injuries resulting from angioplasty, endarterectomy, reduction atherectomy or anastomosis of a vascular graft.

Description

COMPOSITION FOR INHIBITING INTIMAL HYPERPLASIA USING PDGF ANTAGONISTS AND HEPARIN

Government Support

This invention was made with government support under grant number NIH HL 30946 awarded by the National Institutes of Health. The government has certain rights in the invention.

Technical Field

The present invention relates to methods for inhibiting intimal hyperplasia, including restenosis, in a mammal following vascular injury, and to compositions useful within those methods.

Background of the Invention

Proliferation of smooth muscle cells (SMCs) in the vessel wall is an important event in the formation of vascular lesions in atherosclerosis, after vascular reconstruction or in response to other vascular injury. For example, treatment of atherosclerosis frequently includes the clearing of blocked vessels by angioplasty, endarterectomy or reduction atherectomy, or by bypass grafting, surgical procedures in which atherosclerotic plaques are compressed or removed through catheterization (angioplasty), stripped away from the arterial wall through an incision (endarterectomy) or bypassed with natural or synthetic grafts. These procedures remove the vascular endothelium, disturb the underlying intimal layer, and result in the death of medial SMCs. This injury is followed by medial SMC proliferation and migration into the intima, accompanied by excessive deposition of extracellular matrix. This lesion development characteristically occurs within the first few weeks and up to six months after injury and stops when the

balloon injury but may regulate SMC migration into the intima. Platelets are now known to release a number of growth factors, including PDGF, epidermal growth factor (EGF), transforming growth factors alpha and beta (TGFα and TGFβ), insulin-like growth factor I (IGF-I) and platelet derived endothelial cell growth factor, as well as several chemoattractant molecules. Although certain studies implicate PDGF in processes associated with lesion development, no studies have shown the participation of PDGF in these processes in primates.

Removal of atherosclerotic plaques by angioplasty or endarterectomy has limited efficacy, and no effective treatment for restenosis of treated vessels or stenosis of bypass grafts has been developed. There is therefore a need in the art for methods of reducing or preventing the development of SMC-rich lesions in vascular walls, including stenosis of blood vessels following vascular injury, such as injury due to balloon catheterization, endarterectomy or reduction atherectomy, as well as in vascular grafts, organ transplants and catheter emplacements. The present invention provides such methods and fulfills other, related needs.

Disclosure of the Invention

The present invention provides methods and compositions for inhibiting intimal hyperplasia in the vasculature of a mammal, particularly a primate. Examples of intimal hyperplasia include restenosis following angioplasty, endarterectomy or other procedures whereby atherosclerotic plaques are removed from blood vessels. The methods of the invention generally comprise administering to a mammal an effective amount of an anti-growth factor receptor antibody to inhibit intimal hyperplasia. Suitable anti-growth factor receptor antibodies include antibodies to fibroblast growth factor (FGF) receptors, transforming growth factor beta (TGF-β) receptors, insulin-like growth factor I (IGF-I) receptors,

Within another aspect of the invention, intimal hyperplasia in the vasculature of a mammal is inhibited by coordinately administering to the mammal a PDGF antagonist and heparin in respective amounts of antibody and heparin sufficient to combinatorially inhibit the hyperplasia. The antagonist and heparin are administered concurrently or, alternatively, sequentially with either the antagonist or heparin administered first, and the nonadministered remainder of the antagonist and heparin administered within an effective time period thereafter. Within one embodiment, the PDGF antagonist is a non-peptidic PDGF antagonist. Within another embodiment, the PDGF antagonist is an anti-growth factor receptor antibody, such as an anti-PDGF receptor antibody,

Within related embodiments, the coordinately administered antibody or other PDGF antagonist and heparin combinatorially inhibit one or more of the intimal hyperplastic processes of vascular smooth muscle cell proliferation, vascular smooth muscle cell migration, and/or neointimal deposition of extracellular matrix. Within further embodiments, the antibody is either an anti-PDGF-alpha receptor antibody, an anti-PDGF-beta receptor antibody, or a panel of anti-PDGF receptor antibodies. Within additional embodiments, the antibody or other PDGF antagonist inhibits a receptor function of the growth factor receptor, such as binding of the receptor to a receptor ligand, or dimerization of the growth factor receptor. Within other embodiments, an anti-PDGF antibody or other PDGF antagonist is administered which inhibits binding of one or more of the AA, AB and BB isoforms of PDGF to PDGF receptors. Within other embodiments, the heparin comprises a heparan sulfate or a low molecular weight heparin characterized by having a reduced anti-thrombotic activity.

Within other aspects of the invention, an anti-PDGF receptor antibody or other PDGF antagonist and heparin are coordinately administered to a mammal

These and other aspects of the invention will become evident upon reference to the following detailed description and the attached drawings.

Brief Description of the Drawings

Figure 1 illustrates the binding of anti-PDGF receptor monoclonal antibodies to cells that express recombinant PDGF-beta receptor. Results are expressed as mean cpm bound of ^{125}I-rabbit anti-mouse IgG for triplicate determinations. The bars indicate standard deviation.

Figure 2 illustrates the binding of anti-PDGF receptor monoclonal antibodies to cells that express recombinant PDGF-alpha receptor. Results are expressed as mean cpm bound of ^{125}I-rabbit anti-mouse IgG for triplicate determinations. The bars indicate standard deviation.

Figures 3A-3C illustrate the inhibition of PDGF mitogenic activity on human dermal fibroblasts by anti-PDGF receptor monoclonal antibodies. The results are presented as the mean level of [^{3}H]thymidine incorporation for each of the PDGF ligand test conditions. Standard deviation is shown by the T at the top of each bar. Each panel also shows a standard curve for PDGF ligand alone. A) PDGF-AA stimulation, B) PDGF-AB stimulation, C) PDGF-BB stimulation.

Figure 4 illustrates the inhibition of PDGF-AA mitogenic activity on baboon smooth muscle cells by anti-PDGF receptor monoclonal antibodies. A standard curve for ligand alone is shown on the left. Results are presented as the mean level of [^{3}H] thymidine incorporation. Standard deviation is shown by the T at the top of each bar.

Figure 5 illustrates the inhibition of PDGF-AB mitogenic activity on baboon smooth muscle cells by anti-PDGF receptor monoclonal antibodies. A standard curve for

the production (deposition) of extracellular matrix. See, in general, (Harker, Am. J. Cardiol. 60:20B-28B, 1987; DeFeudis, Drug News and Perspectives 5:49-51, 1992). This proliferative process is also manifested in the occlusion of vascular grafts (both natural, including autologous and allogeneic, and synthetic), and in transplanted organs. This proliferative process results in the development of lesions rich in smooth muscle cells and is refered to herein as intimal hyperplasia.

The present invention provides methods for inhibiting the development of SMC-rich lesions through the use of antibodies against growth factor receptors, preferably PDGF receptors, and through the use of other PDGF antagonists, particularly non-peptidic PDGF antagonists. The term "non-peptidic" refers to compounds other than proteins or other peptide-bonded multimers. Such lesions result in the partial or complete blocking of a blood vessel through intimal thickening (hyperplasia). Inhibition of intimal hyperplasia will be understood to include interfering with the proliferative process by reducing or preventing one or more hyperplastic processes, including cell migration, cell proliferation, and extracellular matrix formation. By blocking proliferation and/or migration through interfering with the interaction of PDGF and its receptors, SMC proliferation and subsequent matrix deposition may be reduced. A reduction in intimal hyperplasia is clinically manifested as a significant decrease in loss of lumenal volume after an acute vascular injury. Such a reduction will generally result in a decreased need for re-vascularization procedures (e.g., repeat angioplasty) at the site of the initial injury.

The methods of the present invention are particularly useful in the treatment of intimal hyperplasia due to acute vascular injury. Acute vascular injuries are those which occur rapidly (i.e. over days to months), in contrast to chronic vascular injuries (e.g.

sufficient to cause an immune response. It is preferred to administer the growth factor receptor in combination with an adjuvant, such as Freund's adjuvant, in order to enhance the immune response. Although a single injection of antigen may be sufficient to induce antibody production in the animal, it is generally preferred to administer a large initial injection followed by one or more booster injections over a period of several weeks to several months. See, e.g., Hurrell, J.G.R., ed., Monoclonal Hybridoma Antibodies: Techniques and Applications, CRC Press Inc., Boca Raton, FL, 1982, which is incorporated herein by reference. Blood is then collected from the animal and clotted, and antibodies are isolated from the serum using conventional techniques such as salt precipitation, ion exchange chromatography, affinity chromatography or high performance liquid chromatography.

Within one embodiment of the invention, monoclonal antibodies are used. Monoclonal antibodies provide the advantages of ease of production and lower therapeutic doses as compared to polyclonal antisera, since only antibodies of the desired specificity are used. Methods for producing monoclonal antibodies are well known in the art and are disclosed, for example, by Kohler and Milstein (Nature 256: 495, 1975; Eur. J. Immunol. 6: 511-519, 1976). See also Hurrell, J.G.R., ed., Monoclonal Hybridoma Antibodies: Techniques and Applications, CRC Press Inc., Boca Raton, FL, 1982 and Hart, U.S. Patent No. 5,094,941. As will be appreciated by those skilled in the art, antibody fragments, such as Fab fragments, may also be used.

It is generally preferred to use antibodies that are syngenesious with the patient or that contain syngenesious constant regions. For this reason, genetically engineered antibodies that contain human framework structures will generally be used in the treatment of humans. Methods for producing recombinant human antibodies or humanized non-human (i.e. chimeric)

4917-4921, 1989) and "beta receptor" (Claesson-Welsh et al., Mol. Cell. Biol. 8: 3476-3486, 1988; Gronwald et al., Proc. Natl. Acad. Sci. USA 85: 3435-3439, 1988). In the presence of PDGF ligand, the receptor polypeptides dimerize. Three receptor subtypes are thus possible: $\alpha\alpha$, $\alpha\beta$ and $\beta\beta$. The β receptor is specific for the B-chain of PDGF, while the α receptor binds the A-chain and the B-chain. Consequently, the growth regulatory responsiveness of cells to PDGF depends not only on the availability of PDGF AA, AB and BB ligand isoforms, but also on the expression and availability of different PDGF receptor subtypes (Heldin et al., Cell Regul. 1: 555-566, 1990). Human smooth muscle cells express both α and β receptor subtypes (Heldin et al., Cell Regul. 1: 555-566, 1990), but other cell types are known which express only a single receptor subtype (Gronwald et al., J. Biol. Chem. 264: 8120-8125, 1989).

The anti-PDGF receptor antibodies used within the present invention will preferably be a panel of antibodies capable of inhibiting all three PDGF receptor isoforms ($\alpha\alpha$, $\beta\beta$ and $\alpha\beta$). As used herein, the term "panel" denotes a combination of two or more antibodies having different specificities. The antibodies may be specific for different antigens or for different epitopes on a single antigen. Monoclonal antibodies (MAbs) are preferred.

As noted above, antibodies used within the present invention interfere with the interaction of PDGF and its receptors. In preferred embodiments of the invention, anti-PDGF receptor antibodies are employed which inhibit binding of a PDGF ligand to a PDGF receptor, although those skilled in the art will recognize that the advantages of the invention can also be realized using antibodies that inhibit other receptor-ligand interactions, such as receptor dimerization.

Anti-receptor monoclonal antibodies may also be used as targeting agents for the delivery of compounds of

doses are calculated as described below, taking into consideration affinity and specific activity.

An "antihyperplastically effective amount" of an anti-PDGF receptor antibody or other PDGF antagonist is defined as an amount sufficient to measurably reduce or prevent intimal hyperplasia in a blood vessel, vessel graft or vascular component of a transplanted organ. More specifically, "inhibition of intimal hyperplasia" is herein defined to include any measurable inhibition of one or more of the intimal hyperplastic processes described in the art as vascular smooth muscle cell (VSMC) migration, VSMC proliferation, and neointimal deposition of extracellular matrix. In this context, reduction or prevention of intimal hyperplasia, or of a hyperplastic process involved in intimal hyperplasia, can be readily evaluated using *in vitro, in vivo* and *ex vivo* assay systems known in the art, in particular primate-based assay systems (e.g., non-human or human primate VSMC cultures or vascular tissue explants, or non-human primate *in vivo* tests). In interpreting *in vitro* dosage data, it will be appreciated that different test cells and tissues may express different levels and/or types of PDGF receptors. In addition, cell culture passage number (i.e. number of cell generations elapsed following dissociation or outgrowth of VSMCs from a vascular tissue source) will be recognized as potentially having an important impact on mitogenic and other growth-related activities observed in experimental systems. Similarly, a number of variables must be considered in extrapolating *in vivo* data from non-human systems to estimate antihyperplastic effectiveness of antibodies in humans. In particular, it is important to consider any differences in the nature and severity of a blood vessel injury between experimental and clinical systems to best utilize model data in determining actual treatment protocols for humans. Likewise, interspecies differences in vascular anatomy and histology, and intrinsic differences in the hyperplastic processes

angioplasty for the generation of a vascular lesion mimics a procedure that is commmonly used to re-establish blood flow in stenosed coronary arteries and which leads to restenosis in 30-40% of treated individuals. This model is therefore particularly well suited for testing the use of anti-PDGF receptor antibodies or other PDGF antagonists, alone or in conjunction with heparin.

Another suitable model for testing the efficacy of PDGF antagonist therapy is a baboon model of carotid endarterectomy. In this model an acute injury is made to the medial area of the artery, which subsequently leads to the development of an intimal lesion (Hanson et al., Hypertension 18:I170-I176, 1991). This model mimics the use of carotid endarterectomy to open carotid arteries in humans that have decreased blood flow due to advanced atherosclerosis. A third model for testing the use of PDGF antagonist therapy is a baboon vascular graft emplacement model. It has been demonstrated that the placement of vascular grafts leads to the generation of hyperplastic lesions at the site of the graft (Kraiss et al., J. Clin. Invest. 92:338-348, 1993). These lesions have characteristics similar to those of hyperplastic lesions in humans at sites of vascular injuries.

To test the efficacy of PDGF antagonist therapy in humans, various types of analysis can be used. These include monitoring for a loss in mean lumenal diameter (MLD) by angiography at the 3-6 month period following vascular treatment. Alternative methods to monitor efficacy include intravascular ultrasound, B-mode ultrasound and magnetic resonance imaging. Clinical correlates can also be used to monitor for efficacy of the anti-PDGF receptor antibody treatment. These include a decrease in myocardial infarcts and recurent angina, and the need for repeat re-vascularization.

Antibody dosage levels are calculated from inhibition data after determining clearance of antibody from the blood. In general, dosage is selected with the

initial injury. The antibody may be given via multiple routes including intravenous, intramuscular or subcutaneous injections. In addition the antibody may be delivered locally to the site of vascular injury using perfusion balloon catheters, coating onto stents, or placement on gel coated balloons. In the latter cases it would be expected that the doses of antibody would be substantially less than that required when given systemically. The antibodies may also be delivered by slow-release delivery systems, including such systems incorporated into vascular grafts or stents, or by way of perfusion or double balloon catheters. For inhibition of stenosis in vascular grafts, anti-PDGF receptor antibodies may be covalently attached to the graft through their constant regions or incorporated into the graft in slow-release formulations. Pumps and other known delivery systems may also be employed. In any event, administration is designed to provide the desired daily dose (e.g., a five-day bolus of 25 mg/kg to provide 5 mg/kg/day). Mode and timing of administration of other PDGF antagonists can be determined from chemical and physical properties of the specific antagonist and pharmacokinetic data according to accepted principles.

For use within the present invention, anti-PDGF receptor antibodies are formulated into injectable compositions according to conventional procedures and packaged in sterile containers. The antibodies may be combined with a suitable diluent such as sterile saline or sterile water. The antibody compositions may further contain carriers, stabilizers and excipients such as sugars (e.g. mannitol) or albumin. In the alternative, the antibodies may be provided in lyophilized form and reconstituted in a suitable diluent prior to use. These compositions may be packaged in single or multiple dosage form, for example in sealed ampoules or vials. Non-peptidic PDGF antagonists may be delivered parenterally or enterally (e.g., orally).

these particular heparin examples, more specific heparin subtypes are also known. For example, heparan sulfate moieties produced by endothelial cells (Castellot et al., J. Cell. Biol. 90: 372-379, 1981) and smooth muscle cells (Fritze et al., J. Cell. Biol. 100: 1041-1049, 1985) have been isolated which are reportedly up to 40 times more active than unfractionated heparin for inhibiting proliferation of smooth muscle cells. In addition, among the naturally occurring heparin size variants, fractionated heparin species that exhibit predominantly either anticoagulant or antiproliferative activity have been isolated (Wolinsky et al., J. Am. Coll. Cardiol. 15: 475-481, 1990). The latter activity tends to be present in the low molecular weight heparin species, such as heparins in the range of penta- to decasaccharides, which have been reported to also provide greater bioavailability and a longer half-life (Id., Bacher et al., Thrombosis Res. 70: 295-306, 1993), and may therefore be particularly useful within specific embodiments of the invention. Also included within the definition of heparin for the purposes of describing the invention are synthetic heparins and heparin derivatives, a variety of which have been produced using conventional chemical synthetic, modifying and degradative techniques (see for example, Roden, L. The Biochemistry of Glycoproteins and Proteoglycans (Lennarz, W.J., ed.) pp 267-371, Plenum Publishing Corp., New York, 1980, incorporated herein by reference). The term "low molecular weight heparin having reduced antithrombotic activity" is used to indicate low molecular weight forms having reduced antithrombotic activity (as determined by standard assays) compared to unfractionated heparin.

To determine combinatorially effective doses of antibody/antagonist and heparin, and/or to evaluate combinatorially effective time periods for separately or sequentially administering a PDGF antagonist and heparin, the same general methods described above for assaying antihyperplastic activity of anti-PDGF receptor

level of combinatorial inhibition. In other circumstances, the form or dosage of antagonist, or the timing or mode of administration of antagonist may be imposed by extrinsic circumstances, in which case the heparin administration regimen may need to be coordinately adjusted. For example, in circumstances where a prolonged antibody treatment regimen is desired, lower antibody doses, or less immunogenic antibody forms (e.g., mouse/human chimeric antibodies) may be used to optimize results. In such cases, a coordinate adjustment can be made with respect to the type, dose or timing of administered heparin to achieve a strong, combinatorial antihyperplastic effect.

Using the coordinate antibody and heparin administration methods of the invention, dosages of antibody or other antagonist and heparin in particular may be coordinately varied across a broad range while maintaining a high level of combinatorial inhibition of intimal hyperplasia. This feature of the invention is especially useful for accomodating clinical applications where a low dose of one or the other antihyperplastic agent (i.e. the antagonist or heparin) is desired, such as in cases where dose-limiting toxicities, allergies or other complications are present. Within the methods of the invention, coordinately administered anti-PDGF receptor antibodies and heparin have been found to be combinatorially effective in antibody:heparin dose ratios (i.e. ratio of unit antibody dose to unit heparin dose, by weight) ranging between .001:1 to 1,000:1, and broader. In other words, a unit dose of antibody as low as 1/1,000 of a dose of coordinately administered heparin yields a combinatorially inhibitory effect, while antibody doses 1,000 fold greater than a coordinately administered heparin dose also yields a combinatorial effect. This generally inverse-proportional co-variability of antibody and heparin doses provides extreme flexibility for implementing alternative, coordinate administration

Anti-PDGF receptor antibodies and heparin are preferably administered parenterally, such as by bolus injection or infusion (intravenous, intramuscular, intraperitoneal or subcutaneous) prior to surgery (generally within 24 hours before surgery) and optionally continuing after surgery at intervals of from several hours to several days over the course of one to two weeks or more. Within one embodiment, the antibody is administered as a bolus injection or infusion on the first day of treatment in an amount suficient to provide a minimum circulating level of antibody throughout the intitial, three-day treatment period of between approximately 20 μg and 1 mg/kg body weight. In this regard, it is preferred to use antibodies having a circulating half-life of at least 12 hours, preferably at least 4 days, more preferably up to 14-21 days. Chimeric and humanized antibodies are expected to have circulatory half-lives of up to four and up to 14-21 days, respectively. In many cases it will be preferable to administer daily doses during a hospital stay, followed by less frequent bolus injections during a period of outpatient treatment. The antibodies and heparin may also be delivered by slow-release delivery systems, including such systems incorporated into vascular grafts or stents, or by way of perfusion or double balloon catheters. Pumps and other known delivery systems may also be employed for continuous infusion. Dosing regimens may be varied to provide the desired circulating levels of antibody and heparin based on the pharmacokinetics of these agents. Thus, doses will be calculated so that the desired circulating levels of therapeutic agents are maintained. Daily doses referred to above may be administered as larger, less frequent bolus administrations to provide the recited dose averaged over the term of administration. Non-peptidic PDGF antagonists may be administered enterally.

7 demonstrates the inhibition of PDGF mitogenic activity on human dermal fibroblasts using anti-PDGF receptor monoclonal antibodies. Example 8 demonstrates the inhibition of PDGF mitogenic activity on baboon smooth muscle cells using anti-PDGF receptor monoclonal antibodies. Examples 9 and 10 disclose the use of anti-PDGF receptor monoclonal antibodies to inhibit baboon serum mitogenic activity. Example 11 demonstrates the inhibition of baboon aortic smooth muscle cell migration by anti-PDGF receptor monoclonal antibodies. Example 12 demonstrates the ability of anti-PDGF receptor MAbs to inhibit PDGF activity up to eight hours after the ligand has bound to receptors. Example 13 discloses the displacement of receptor-bound PDGF from human osteosarcoma cells by anti-PDGF receptor MAbs. Example 14 demonstrates the inhibition of PDGF and baboon serum mitogenic activity on vascular smooth muscle cells using anti-PDGF receptor monoclonal antibodies administered alone or coordinately administered with heparin. Example 15 discloses the use of heparin, alone or coordinately administered with anti-PDGF receptor monoclonal antibodies, to inhibit serum mitogenic activity on baboon vascular smooth muscle cells. Example 16 discloses further studies demonstrating inhibition of serum mitogenic activity on baboon smooth muscle cells using anti-PDGF receptor monoclonal antibodies coordinately administered with heparin. Examples 17 and 18 disclose studies comparing the antimitotic activities of parent murine and mouse/human chimeric anti-PDGF-alpha and beta receptor antibodies coordinately administered with heparin. Example 19 further describes the inhibitory activity of coordinately administered heparin and anti-PDGF receptor antibodies against serum mitogenic activity. Example 20 demonstrates the inhibition of smooth muscle cell outmigration from baboon aortic explants by anti-PDGF receptor monoclonal antibodies ccordinately administered with heparin. Examples 21-23 disclose binding studies of

the PDGF-alpha receptor or PDGF-beta receptor were prepared essentially as disclosed in U.S. Patent No. 5,155,027; U.S. Patent Application Serial No. 07/634,510 and EP 325,224, which are incorporated herein by reference in their entirety. In one case mouse myeloma cells were transfected with cDNAs for both heavy-chain and light-chain/PDGF receptor extracellular domain fusion proteins. These cells secrete into their culture media a molecule which is analogous to human IgG in that it is composed of two light-chain and 2 heavy-chain fusion proteins. This compound is designated as tetrameric IgG/PDGFr. In another case a cDNA for light-chain/PDGF receptor extracellular domain fusion protein was transfected into the cells alone. These cells secrete monomeric light-chain fusion proteins into their culture media, designated as monomeric IgG/PDGFr. The alpha- and beta-receptor fusion proteins were designated IgG/PDGFr-alpha (tetrameric) and IgG/PDGFr-beta (monomeric and tetrameric), respectively. The fusion proteins were purified by either immunoaffinity purification using anti-PDGF receptor monoclonal antibodies, or by protein A-Sepharose™ chromatography.

Example 1

Preparation of PDGF Receptor Monoclonal Antibodies

Fusion proteins comprising an IgG constant region joined to the extracellular domain of either the PDGF-alpha receptor (PDGFr-alpha) or the PDGF-beta receptor (PDGFr-beta) were prepared essentially as disclosed in U.S. Patent No. 5,155,027, which is incorporated herein by reference in its entirety. The alpha and beta receptor fusions were designated IgG/PDGFr-alpha and IgG/PDGFr-beta, respectively. The monomeric IgG/PDGFr-beta was expressed as a fusion of a human kappa light chain constant region and the PDGF-beta receptor extracellular domain. The tetrameric IgG/PDGFr-beta was

antibodies. Cell fusions were designated by number (e.g., 162, 163).

TABLE 1

NS-1 Medium

For a 500 ml solution:

5 ml 10 mM MEM non-essential amino acids (GIBCO BRL, Gaithersburg, MD)
5 ml 100 mM sodium pyruvate (Irvine, Santa Ana, CA)
5 ml 200 mM L-glutamine (GIBCO BRL)
5 ml 100x Penicillin/Streptomycin/Neomycin (GIBCO BRL)
75 ml inactivated fetal calf serum (BioCell, Carson, CA)
1 gm $NaHCO_3$

Add RPMI 1640 (GIBCO BRL) to a total volume of 500 ml. Sterilize by filtration through a 0.22 lm filter.

100x HT Stock

38.5 mg thymidine
136.10 mg hypoxanthine

Dissolve the thymidine and hypoxanthine in distilled H_2O and bring volume up to 100 ml. Warm the solution to 60-70°C to dissolve the solids. After the solids have dissolved, readjust the volume to 100 ml. Sterilize by filtration through a 0.22 µm filter. Store frozen at -20°C.

1000x A Stock

17.6 ng aminopterin

Add sterile distilled water to the aminopterin and bring the volume to 50 ml. Add 1 N NaOH drop-wise until the aminopterin dissolves. Bring the final volume to 100 ml with distilled H_2O. Sterilize by filtration through a 0.22 lm filter. Store frozen at -20°C.

Table 1. continued

Binding Media

500 ml Ham's F-12 (GIBCO BRL)
12 ml 1 M Hepes pH 7.4
5 ml 100x Penicillin/Streptomycin/Neomycin (GIBCO BRL)
1 gm rabbit serum albumin (Sigma)

Mito Media

For a 500 ml solution:
250 ml DMEM (GIBCO BRL)
250 ml Ham's F-12 (GIBCO BRL)
0.25 ml 10 mg/ml stock of insulin (GIBCO BRL) to give a final concentration of 5 µg/ml
1 ml 10 mg/ml stock of transferrin (Collaborative Research, Bedford, MA) to give a final concentration of 20 µg/ml
2 ml 4 µg/ml stock of selenium (Aldrich Chemical, Milwaukee, WI) to give a final concentration of 5 nM
5 ml 10% stock solution of bovine serum albumin (GIBCO BRL)to give a final concentration of 0.1%.

Example 2

Identification and Characterization of Hybridomas Producing Antibodies to the PDGF Beta Receptor

Hybridomas from cell fusion 162 were tested for the production of antibodies to the PDGF-beta receptor. Assays used for identification of positive hybridomas included enzyme linked immunosorbent assays (ELISA), inhibition of ^{125}I-PDGF-BB binding to IgG/PDGFr-beta, and inhibition of ^{125}I-PDGF-BB binding to human dermal fibroblasts.

The ELISA assays were carried out in 96-well microtiter plates which had been coated with monomeric IgG/PDGFr-beta. To coat the wells, IgG/PDGFr-beta was

The plates were washed with ELISA C buffer, then incubated for 1.5 hours with tetrameric IgG/PDGFr-beta, and diluted in ELISA B buffer to a final concentration of 25 ng/ml. The wells were washed with ELISA C buffer to remove unbound IgG/PDGFr-beta.

Hybridoma supernatants were pooled in groups of two, and 100 µl of the pooled samples was added to each of the microtiter wells. The wells were incubated for 1 hour at 37°C. To each well was then added 50 µl of ^{125}I-PDGF-BB (approximately 50,000 cpm per well). After a 1 hour incubation at 37°C the wells were washed three times with binding media (Table 1). 100 µl of 0.1M NaCitrate, pH 2.5, was added to the wells for 5 minutes at room temperature, the solution was harvested and transfered to 12x75 mm tubes, and the tubes were counted in a gamma counter to determine the level of ^{125}I-PDGF-BB binding. Antibodies which bound to IgG/PDGFr-beta and blocked ^{125}I-PDGF-BB binding were detected by a decrease in the level of ^{125}I-PDGF-BB bound, as compared to culture media alone.

Pools of media that were determined to be positive for IgG/PDGFr-beta neutralizing antibody were rescreened using an assay format similar to that described above to identify the individual wells which contained hybridomas producing the neutralizing antibody.

Media samples from culture wells that were positive either by ELISA or by inhibition of ^{125}I-PDGF-BB binding were subsequently assayed in a down-regulation assay format (Hart et al., J. Biol. Chem. 262: 10780-10785, 1987) for the ability to recognize PDGF-beta receptor on human dermal fibroblasts. The binding of PDGF-BB to the PDGF-beta receptor at 37°C leads to the internalization of the receptors from the cell surface and a subsequent decrease in the number of cell-surface receptors, a phenomenon refered to as down-regulation. The fibroblasts were plated into 96-well culture dishes at 10,000 cells per well and maintained in culture media for 1-2 days prior to use. To one set of wells was added

assays were carried out in 96-well microtiter plates. The plates were initially coated with goat anti-human IgG, 2 μg/ml in ELISA A buffer, for 2 hours at 37°C. The plates were washed with ELISA C buffer, then incubated for 1 1/2 hours at 37°C with ELISA B buffer to block nonspecific binding sites. The plates were washed with ELISA C buffer, then either used immediately or left for 1-4 days at 4°C until use. At the time of the assay the plates were washed once with ELISA C buffer, then incubated for 1 1/2 hours at 37°C with tetrameric IgG/PDGFr-beta diluted to 25 ng/ml in binding medium. The plates were then washed with ELISA C buffer to remove unbound IgG/PDGFr-beta.

Hybridoma supernatants were pooled in groups of two wells, and 100 μl of the pooled samples was added to each of the microtiter wells. The plates were incubated for 1 hour at 37°C, then washed with binding medium. To the wells was added horseradish peroxidase-conjugated goat anti-mouse IgG (Tago, Burlingame, CA) diluted 1:1000 with binding medium. The wells were incubated for 1 hour at 37° C, then washed with binding medium to remove unbound HRP-conjugated goat anti-mouse IgG. ^{125}I-PDGF-BB, aproximately 26,000 cpm/well, was then added to the wells for an additional 1 hour at 37°C. The wells were washed with binding medium, then incubated with reaction buffer for development of the ELISA. The reaction was stopped by the addition of 100 μl/well of 1 N H_2SO_4, and the plates were read in a Dynatech ELISA plate reader using a filter to monitor the absorbance at 490 nm.

The contents of the wells were then transfered to 12x75 mm test tubes, and the samples were counted in a gamma counter to measure the level of ^{125}I-PDGF-BB binding.

The above-described assay identified hybridoma cultures producing antibody to IgG/PDGFr-beta by ELISA, as well as by the ability to block the binding of ^{125}I-PDGF-BB to tetrameric IgG/PDGFr-beta. Those pooled samples that were positive were subsequently reassayed using the

Example 4

Characterizaton of Anti-PDGF Beta Receptor MAbs 162.62 and 163.31.

MAbs 162.62 and 163.31 (produced from hybridoma clones 162.62 and 163.31, respectively) were compared for the ability to block the binding of ^{125}I-PDGF-BB to either tetrameric IgG/PDGFr-beta or to PDGF-beta receptor on human dermal fibroblasts. Inhibition of ^{125}I-PDGF-BB binding to IgG/PDGFr-beta was tested essentially as described above for the intital screening of fusion 163. Instead of adding conditioned culture media, known amounts of antibody diluted in NS-1 medium were added simultaneously with ^{125}I-PDGF-BB to the IgG/PDGFr-beta coated wells. NS-1 medium alone was used as a negative control. The addition of PDGF-BB, 500 ng/ml, to NS-1 medium was used to determine the level of nonspecific binding by ^{125}I-PDGF-BB. The wells were incubated at 4°C for 2 1/2 hours, then washed with PBS. 100 µl of 0.1M citrate pH 2.5 was added to each well to remove the bound ^{125}I-PDGF-BB, the samples were transferred to 12x75 mm tubes, and the tubes were then counted in a gamma counter.

To assay binding to human dermal fibroblasts, the fibroblasts were plated at approximately 20,000 cells/well in 24-well culture dishes. The cells were used for assay 2-7 days after plating. The antibodies were diluted in binding media to the concentrations shown in Table 2, then mixed with ^{125}I-PDGF-BB, and 0.5 ml aliquots were added to duplicate wells of fibroblasts. Binding media alone was used as the negative control, and the addition of 500 ng/ml of PDGF-BB was used to determine nonspecific binding for ^{125}I-PDGF-BB. The cells were incubated for 2 1/2 hours at 4°C, then washed with binding media to remove unbound ligand. The cells were then incubated with extraction buffer, and the extracts were harvested and counted in a gamma counter.

The results of the binding studies are shown in Table 2. The data are presented as specific cpm bound for

washed, incubated with extraction buffer, and the extracts were counted in a gamma counter to determine the level of ^{125}I-PDGF-BB binding. To determine nonspecific binding, 500 ng/ml of unlabeled PDGF-BB was added during the first incubation step. The results, presented in Table 3, show that the addition of MAb 162.62 led to a 47% displacement of prebound ^{125}I-PDGF-BB. Thus, MAb 162.62 was able to displace receptor-bound PDGF-BB from the surface of human dermal fibroblasts.

Table 3

Ability of MAb 162.62 to Displace Receptor-bound ^{125}I-PDGF-BB From Human Dermal Fibroblasts

1st Inc.	2nd Inc.	CPM Bound	BB Removal
^{125}I-BB	Binding Media	581	
^{125}I-BB	MAb 162.62	308	47 %

The subclass for MAbs 162.62 and 163.31 were determined by ELISA using IgG/PDGFr-beta coated wells and subclass specific secondary antibody. MAb 162.62 was found to be an IgG2b isotype while MAb 163.31 was found to be an IgG1 isotype.

Example 5

Identification and Characterization of Hybridomas Producing Anti-PDGF Alpha Receptor Antibodies

Hybridomas from cell fusion 169 were tested for the production of antibodies to the PDGF-alpha receptor by a combination ELISA/PDGF binding competition assay. These assays were carried out in 96-well microtiter plates. The plates were initially coated with goat anti-human IgG, 2 µg/ml in ELISA A buffer, overnight at 4°C. The plates were washed with ELISA C buffer, then incubated with ELISA B buffer to block nonspecific binding sites. The plates were washed with ELISA C buffer, then incubated overnight

screened using the combination ELISA/^{125}I-PDGF-AA binding competition assay essentially as described above.

To verify that MAbs 169.14 and 169.31 recognize native PDGF-alpha receptor on monolayers of mammalian cells, the two antibodies were analyzed for the ability to block ^{125}I-PDGF-AA binding to alpha T-7 cells. These cells are canine kidney epithelial cells that do not naturally express PDGF-alpha receptor, but have been transfected with a cDNA coding for the full length PDGF-alpha receptor (U.S. Patent No. 5,371,205o. 5,371,205; PCT Publication WO 90/14425). These cells express approximately 100,000 recombinant receptors per cell. The alpha T-7 cells were cultured in 96-well plates to approximately 95% confluency. The culture medium was removed, and dilutions of MAbs 169.14 and 169.31 were added to the cells. Controls were NS-1 medium, and NS-1 medium containing 500 ng/ml of PDGF-BB to determine the nonspecific binding component for ^{125}I-PDGF-AA. To each well was added 100 µl of the test sample plus 10 µl of ^{125}I-PDGF-AA (approximately 22,000 cpm per well). The cells were incubated with the samples for 2 hours at 4°C, washed with PBS, then extracted with 100 µl/well of extraction buffer. The extracts were harvested and counted in a gamma counter. The results are shown in Table 4. These results demonstrate that these two MAbs recognize membrane-bound PDGF-alpha receptor in mammalian cells in addition to IgG/PDGFr-alpha.

express approximately 500,000 human PDGF-beta receptors per cell. The Alpha 1-10 cells are BHK 570 cells that have been transfected with a cDNA coding for the full length human PDGF-alpha receptor (U.S. Patent No. 5,371,205; PCT Publication WO 90/14425). These cells express approximately 1,000,000 human PDGF-alpha receptors per cell. To demonstrate binding specificity for either the PDGF-alpha or beta receptor, cell surface binding studies using the anti-PDGF receptor MAbs were done with these two cell lines.

Both the Clone 8 and Alpha 1-10 cells were cultured in 24-well plates to confluency. PDGF-BB (200 ng/ml) was added to one-half of the cells to stimulate PDGF receptor down-regulation, and vehicle control (10 mm acetic acid, 0.25% rabbit serum albumin) was added to the other half. The cells were incubated for 1-2 hours at 37° C, then washed with PBS chilled to 4°C. Purified MAbs 162.62, 163.31, 169.14 and 169.31, diluted to 5 μg/ml in binding medium, were added to triplicate wells of the PDGF-BB-treated and nontreated control cells. The cells were incubated for approximately 2 hours on ice, then washed with chilled PBS to remove unbound antibody. The test wells were then incubated on ice for 30 minutes with ^{125}I-labeled rabbit anti-mouse IgG, diluted in binding medium to approximately 400,000 cpm/well. The wells were washed with PBS, then incubated with extraction buffer. The extracts were harvested and counted in a gamma counter. The results, shown in Figure 1, demonstrated that only MAbs 162.62 and 163.31 bound specifically to the PDGF-beta receptor, as demonstrated by the significant decrease in binding to the PDGF-BB treated Clone 8 cells when compared to untreated controls. The high level of binding by MAb 169.14 was due to an elevated level of nonspecific binding by this antibody, because there was no significant decrease in ^{125}I-rabbit anti-mouse IgG binding to the PDGF-BB treated cells. In contrast, only MAbs 169.14 and 169.31 showed binding to the PDGF-alpha

80% by MAb 162.62, and greater than 92% by MAb 169.14 or the antibody pool (Figure 3B). In contrast, activity of PDGF-BB was only minimally inhibited by MAb 169.14, but was inhibited approximately 80% by MAb 162.62 and greater than 92% by the antibody pool (Figure 3C).

These results are consistent with the model of PDGF ligand binding which describes that PDGF-AA binds to PDGF-alpha/alpha receptor dimers, PDGF-AB binds to PDGF-alpha/alpha and -alpha/beta receptor dimers and PDGF-BB binds to all three PDGF receptor dimers; -alpha/alpha, -alpha/beta and -beta/beta (reviewed in Hart et al., J. Invest. Derm. 94: 535-575, 1990). Thus, if MAb 169.14 binds to and inhibits PDGF binding to the alpha receptor, then it would be expected to inhibit essentially 100% of PDGF-AA and AB mitogenic activity, since alpha receptor binding is required for both of these ligands. This model is consistent with the results described above. The binding to and the inhibition of the PDGF-beta receptor by MAb 162.62 would then be expected to limit the amount of PDGF-AB and BB mitogenic to a level that is consistent with PDGF-AA, since AB and BB would only be able to bind to alpha/alpha dimers. Again, this is consistent with the findings of the study descibed above.

In summary, anti-PDGF-receptor MAbs 162.62 and 169.14 are able to inhibit the mitogenic activity of the three forms of PDGF in manners that are consistent with the current hypothesis as to PDGF receptor binding by the three PDGF ligands. Additionally, the use of the two antibodies in conjunction is able to inhibit essentially 100% of the PDGF mitogenic activity on human dermal fibroblasts.

were incubated between 2-4 hours at 37°C, washed with PBS, then harvested with trypsin and counted for [^{3}H]thymidine incorporation in a Betaplate™ liquid scintillation counter (Wallac). As shown in Figure 4, PDGF-AA mitogenic activity was 100% inhibited by MAb 169.31 as well as by the antibody pool, but not by MAb 163.31. PDGF-AB mitogenic activity was completely inhibited by both MAbs individually as well as by the antibody pool (Figure 5). It is interesting to note that the level of [^{3}H]thymidine incorporation in the presence of the MAbs was below the level obtained with the addition of vehicle control only. This was similarly seen with MAb 169.31 on the PDGF-AA plate (Figure 4). For the PDGF-BB stimulated cells, MAb 169.31 and MAb 163.31 gave less than 50% inhibition individually, while a pool of the two antibodies was able to inhibit approximately 75% of the PDGF mitogenic activity (Figure 6).

To further demonstrate the inhibitory potency of these antibodies to neutralize the mitogenic activity of PDGF on baboon smooth muscle cells, two anti-PDGF-alpha receptor MAbs, 169.14 and 169.31, were analyzed for the ability to inhibit PDGF-AA mitogenic activity. BVSMCs were plated and treated essentially as described above. To one set of wells were added increasing concentrations of PDGF-AA in order to generate a standard curve of PDGF-AA mitogenic activity (Figure 7A). The PDGF-AA samples ranged from 10 ng/ml down to 0.31 ng/ml. To a second set of wells, a standard dilution of PDGF-AA was added to give a final concentration of 10 ng/ml. Decreasing concentrations of MAbs 169.14 and 169.31 were then added to the wells to monitor the inhibitory potency for each of the MAbs, as determined by a decrease in the level of [^{3}H]thymidine incorporation (Figure 7B). The findings demonstrate that even at 8 ng/ml of antibody, there was greater than 90% inhibition of a 10 ng/ml solution of PDGF-AA.

demonstrate that baboon serum mitogenic activity is minimally inhibited by MAb 169.31, but inhibited greater than 50% by MAb 163.31. The pool of the two antibodies inhibited greater than 75% of the serum mitogenic activity.

These results demonstrate that the majority of the mitogenic activity in baboon serum towards baboon smooth muscle cells can be inhibited through the use of anti-PDGF receptor monoclonal antibodies. Studies by the inventors have shown that the predominant form of PDGF in baboon platelets is PDGF-BB. Due to the large percentage of PDGF-beta receptors on baboon smooth muscle cells, it is consistent that the anti-PDGF-beta receptor MAb would have the largest inhibitory activity towards baboon serum.

Example 10
The Effect of Circulating MAb 169.31 on Baboon Serum Mitogenic Activity

A study was performed to monitor the circulating levels of MAb 169.31 after the administration of a bolus intravenous (i.v.) injection of 25 mg into a baboon. Serum was obtained at various intervals following antibody injection, and the level of circulating antibody was determined by ELISA. Sheep anti-mouse IgG was added to 96-well microtiter dishes in ELISA buffer A at a concentration of 2 µg/ml. The plates were incubated overnight at 4°C, washed with ELISA C buffer, then incubated with ELISA B buffer to block nonspecific binding sites. The plates were washed with ELISA C buffer, then incubated with 100 µl/well of test sample. Baboon plasma or serum containing monoclonal antibody 169.31 was diluted 1:1000 with ELISA B buffer and added to the test wells. Standards, consisting of purified MAb 169.31 spiked into control baboon plasma or serum, were diluted 1:1000, similar to the test plasma/serum samples, then added to the test wells. Standards ranged from 100 ng/ml to 1.56

MAb 169.31 circulating for at least 18 hours in baboon blood retains essentially all of its biological activity for inhibiting baboon serum mitogenic activity on baboon smooth muscle cells.

Example 11
Inhibition of Cell Outgrowth from Baboon Aortic Explants

Anti-PDGF receptor monoclonal antibodies were tested for the ability to decrease the rate of smooth muscle cell outmigration from explants of baboon aortic tissue. The inner media of the thoracic aorta of baboons was dissected out in DMEM culture media containing 10 mM Hepes. The aortic tissue was sectioned into 1 mm square sections, and the explants were placed onto tissue culture flasks. After a 10 minute incubation to allow time for the explants to adhere to the flasks, culture media, DMEM plus 6 μg/ml insulin and 5 μg/ml transferin, was added to the explants, and the samples were incubated at 37°C with 5% CO_2. A total of 15 explants were set up in each culture flask. At various times following the establishment of the explants they were examined under a high power microscope to count the number of explants that had visible cell outgrowth onto the culture dish. Explants were counted as positive if at least one cell migrated from the explant tissue out onto the culture dish surface. Explants were followed for at least seven days. In experiment #1, the explants were cultured in the DMEM culture media supplemented with insulin and transferrin containing the following test samples: 1) Anti-PDGF alpha receptor MAb (169.31) at 50 μg/ml; 2) Anti-PDGF beta receptor MAb (163.31) at 50 μg/ml; or 3) DMEM media alone (control). In experiment #2 the explants were cultured in DMEM plus insulin and transferrin, and either 1) a pool of anti-PDGF alpha and beta receptor MAbs (169.31 and 163.31) at 25 μg/ml each; or 2) DMEM alone (control)

Example 12

Inhibition of PDGF Mitogenic Activity on Human Dermal Fibroblasts by Delayed Addition of Anti-PDGF Receptor Monoclonal Antibodies

Human dermal fibroblasts were plated at approximately 20,000 cells per well in 24-well culture dishes and grown until quiescent in DMEM containing 2% fetal calf serum. The cells were stimulated with either PDGF-AA, AB or BB. Increasing concentrations of each of the PDGF ligands were added to the cells to generate standard curves of mitogenic potency for the three PDGF isoforms. The final PDGF concentrations used for the standards were 5, 2.5, 1.25, 0.62, 0.31, 0.15 and 0.0 ng/ml. 50x stock solutions of PDGF were made in 10 mM acetic acid containing 0.25% rabbit serum albumin. 25 µl of each of the stock solutions were added to triplicate test wells. To look for inhibitory activity by MAbs 162.62 and 169.14, wells containing the fibroblasts were incubated with 5 ng/ml of PDGF, final concentration. At various time intervals following the addition of the PDGF samples (1, 2, 4, 6 and 8 hours), a pooled sample of MAb 162.62 and MAb 169.14, 25 µg/ml final concentration for each MAb, was added to triplicate wells of the cells that had been treated with 5 ng/ml of PDGF. Nine hours after the addition of the PDGF samples, 50 µl of [^{3}H]thymidine, 20 µCi/ml in DMEM containing 1% fetal calf serum, was added to each well. The samples were incubated for an additional 13-15 hours at 37°C. The cells were washed with PBS, then harvested with trypsin and counted in a Betaplate™ liquid scintillation counter (Wallac).

The results, presented in Table 6, are given as mean cpm of [^{3}H]thymidine incorporated +/- standard deviation, for triplicate determinations. The data are given for both the PDGF standard curves, and for the time course of antibody addition. The results demonstrate that

Table 6

PDGF-AA (ng/ml) MAbs 162.62/169.14 (PDGF-AA, 5 ng/ml)

ng/ml	cpm +/-	(st.dev.)	Time in Hrs	cpm +/-	(st.dev.)
5.0	7888	(768)	1	5400	(870)
2.5	7892	(460)	2	4350	(431)
1.25	6044	(1126)	4	5323	(574)
0.62	5569	(315)	6	5300	(768)
0.31	5072	(224)	8	6028	(276)
0.15	4888	(393)			
0.0	4804	(320)			

PDGF-AB (ng/ml) MAbs 162.62/169.14 (PDGF-AB, 5ng/ml)

ng/ml	cpm +/-	(st.dev)	Time in Hrs	cpm +/-	(st. dev.)
5.0	16370	(409)	1	4372	(443)
2.5	16621	(878)	2	4783	(401)
1.25	14061	(1066)	4	4363	(427)
0.62	11238	(238)	6	5238	(611)
0.31	9206	(428)	8	5659	(667)
0.15	8061	(1054)			
0.00	5253	(443)			

PDGF-BB (ng/ml) MAbs 162.62/169.14 (PDGF-BB, 5ng/ml)

ng/ml	cpm +/-	(st.dev)	Time in Hrs	cpm +/-	(st. dev.)
5.0	12427	(1366)	1	2811	(291)
2.5	15445	(977)	2	3076	(169)
1.25	13712	(976)	4	4298	(574)
0.62	11989	(1248)	6	5089	(420)
0.31	9482	(2089)	8	7335	(502)
0.15	6905	(456)			
0.00	3090	(272)			

of the counts. The pool of 169.31 and 162.62 displaced 44% of the prebound ^{125}I-PDGF-BB. These results show that, in addition to being able to block PDGF binding, the anti-PDGF receptor MAbs are also able to displace prebound PDGF-AA and BB from cell-surface receptors.

Table 7

Ability of Anti-PDGF Receptor MAbs to Displace Recptor-bound ^{125}I-PDGF-AA and ^{125}I-PDGF-BB from Human Osteosarcoma Cells

First Inc.	Second Inc.	CPM Bound	% Displacement
^{125}I-PDGF-AA	Binding Media	458 (46)	0
"	169.14	164 (91)	64
"	169.31	174 (58)	62
"	162.62	415 (18)	9
"	163.31	420 (40)	8
"	169.31/162.62	116 (24)	75
^{125}I-PDGF-BB	Binding Media	528 (41)	0
"	169.14	411 (87)	22
"	169.31	395 (30)	25
"	162.62	349 (48)	34
"	163.31	518 (129)	2
"	169.31/162.62	289 (58)	44

Example 14

Inhibition of Baboon Serum and PDGF-BB Stimulated Smooth Muscle Cell Mitogenesis By Anti-PDGFr MAbs Applied Independently or Coordinately With Heparin

Anti-PDGFr-alpha MAb 169.31 and anti-PDGFr-beta MAb 163.31 were analyzed independently, and in coordinate administration assays with heparin, to determine the

adding 50 µl of the stock solutions to appropriate wells to give final PDGF-BB concentrations on the cells of 2, 1 and 0.5 ng/ml.

After addition of the treatments, the cells were incubated for 20 hours at 37°C. Mitogenic stimulation of BVSMCs was assessed by measuring the uptake of [^{3}H]thymidine. 50 µl of a 20 µCi/ml [^{3}H]thymidine stock solution, made up in DMEM, was added directly to the cells for a final concentration of 1 µCi/well. The cells were incubated for 4 hours at 37°C, washed once with PBS, treated with 0.25 ml of trypsin until cells detached, and harvested onto a filter using a cell harvester (LKB Wallac). The filters were counted using a Betaplate™ liquid scintillation counter (Wallac).

The data for antibody and antibody/heparin inhibition of PDGF-BB stimulation are shown in Table 8. Data in Table 8 are presented as mean counts per minute (cpm) of [^{3}H]thymidine incorporated by baboon smooth muscle cells stimulated with PDGF-BB. Values of percent inhibition were determined directly from the measured decrease in incorporation of [^{3}H]thymidine. A dose-response table of PDGF-BB mitogenic activity is included in Table 8. In Experiment #1, the addition of antibody 169.31 to cells stimulated with PDGF-BB caused a marked inhibition in [^{3}H]thymidine incorporation at antibody doses of 1 and 0.1 µg/ml. The administration of heparin to the cells coordinately with antibody 169.31 resulted in a combinatorially effective antimitogenic result, i.e. [^{3}H]thymidine incorporation was inhibited to a greater extent than was measured for either the antibody or heparin administered alone. Analysis of antibody 163.31, Experiment #1, showed that a dose of 25 µg/ml was also able to inhibit [^{3}H]thymidine incorporation, but at a much lower level than observed for antibody 169.31. When heparin was added along with antibody 163.31, a combinatorially effective inhibition was also observed, but this effect was only seen at higher antibody

Table 8

Inhibition of PDGF-BB Mitogenic Activity By Anti-PDGFr MAbs Administered Independently or Coordinately With Heparin

EXPERIMENT 1

MAb (µg/ml)		[^{3}H]Thymidine Incorporation			
163.31	169.31	(-) Heparin	% Inhib.	(+) Heparin	% Inhib.
0	0	4,714	0%	2,543	46%
0	1	2,271	52%		
0	0.1	2,637	44%	1,582	66%
0	0.01	4,078	13%	1,983	58%
0	0.001	4,486	05%	2,311	51%
25	0	3,969	16%	1,844	61%
5	0	4,460	05%	2,484	47%
1	0	4,268	09%	2,559	46%
0.2	0	4,504	04%	2,671	43%
25	1	1,015	78%		
5	1	1,859	61%		
1	1	2,007	57%		
1	0.2	1,979	58%		
1	0.04	2,090	56%		
0	0	4,414	06%		

PDGF-BB Dose-Response

PDGF-BB (ng/ml)	CPM
2.0	4,714
1.0	2,313
0.5	1,588
0.0	161

observed was no greater than the inhibitory activity exhibited by heparin alone. In contrast, the assays involving monoclonal antibody 163.31 showed significant inhibition of [^{3}H]thymidine incorporation at an antibody concentration of 25 μg/ml. Moreover, when heparin was administered coordinately with antibody 163.31, there was a marked combinatorially effective inhibition of mitogenic activity as measured by [^{3}H]thymidine incorporation, i.e. well above the antimitogenic effects observed for either the antibody or heparin alone. This combinatorially effective inhibition was particularly pronounced at MAb 163.31 concentrations between 0.2 μg/ml and 5 μg/ml.

Coordinate administration of 1 μg/ml of antibody 169.31 with increasing doses of antibody 163.31 showed a dose-dependent inhibition of [^{3}H]thymidine incorporation. The coordinate administration of antibody 169.31 with antibody 163.31 also resulted in a pronounced combinatorially effective inhibition of serum mitogenic activity as shown in Table 9.

Data in Table 9 are presented as mean counts per minute (cpm) of [^{3}H]thymidine incorporated by baboon smooth muscle cells stimulated with 1.25 % baboon serum. Values of percent inhibition were calculated from a standard curve generated using the serum dose-response data presented.

Example 15

Inhibition of Serum Mitogenic Activity on Baboon Vascular Smooth Muscle Cells By Heparin Administered Alone, or Coordinately Administered With Anti-PDGFr MAbs

Both unfractionated heparin (UH) (Sigma, St. Louis, MO) and low molecular weight heparin (LMWH) (Logiparin®, Novo Nordisk, Bagsvaerd, Denmark) were evaluated for their ability to inhibit the mitogenic activity of baoon serum on baboon vascular smooth muscle cells. Each form of heparin was administered to the cells independently or coordinately with anti-PDGFr MAbs. The LMWH used for these studies was generated by heparinase treatment of an unfractionated heparin, and is composed of heparin species with an average molecular weight of 5,500 daltons. The LMWH has a decreased antithrombotic activity compared to unfractionated heparin in an APTT assay, estimated at about 50 Units/mg.

To assess the anti-mitogenic activity of the two heparin preparations, with and without coordinately administered anti-PDGFr antibodies, BVSMCs from aortic explants (designated BO54 cells) were plated into 24-well tissue culture dishes at 2.5×10^4 cells per well in DMEM (GIBCO BRL) supplemented with 10% fetal bovine serum. The cells were maintained in this media for three days at 37°C in a 5% CO_2 atmosphere. The media was then replaced with 1 ml/well of Mito Media, and the cells were cultured in this media for an additional 24 hours.

Baboon serum was added to the test wells at a final concentration of 2.5%. This was done by diluting the serum 1:1 with PBS and adding 50 µl of the diluted serum to each well. To analyze heparin preparations for their inhibition of baboon serum-stimulated DNA synthesis in baboon smooth muscle cells, the heparin was added to the cells either independently or coordinately with the two anti-PDGFr monoclonal antibodies. The heparin samples were diluted with PBS to give a 400 µg/ml concentrate,

Table 10

Antibody Treatment	(-) Heparin	(+)UH	(+)LMWH
Control	36,032 +/- 4,512	34,912 +/- 5,617	34,140 +/- 2,667
(% Inhibition)		(3%)	(5%)
Anti-PDGFR	27,005 +/- 2,227	12,377 +/- 3,785	19,967 +/- 974
(% Inhibition)	(25%)	(66%)	(45%)

Data are presented as mean +/- standard deviation for counts per minute (CPM) of [^{3}H]thymidine incorporated by baboon smooth muscle cells stimulated with 2.5% baboon serum. Percent inhibition of cpm incorporated from control value, in the absence of both added heparin and anti-PDGFR antibody, is presented in parentheses. Values of percent inhibition were determined directly from the measured decrease in incorporation of [^{3}H]thymidine, rather than by comparison to a dose-response curve for serum stimulation.

Example 16

Dose-Response of Inhibition of Serum Mitogenic Activity On Baboon Vascular Smooth Muscle Cells By Anti-PDGFr MAbs Coordinately Applied With Unfractionated Heparin or Low Molecular Weight Heparin

A dose-response assay of anti-PDGFr beta monoclonal antibody 163.31, in the presence of a constant amount of anti-PDGFr alpha antibody 169.31 (1 μg/ml), was performed to evaluate concentration dependence of anti-PDGFr MAb inhibition of DNA synthesis in BVSMCs stimulated by baboon serum. This dose-response was evaluated in the absence of any added heparin, as well as in the presence

wells receiving the 25 and 10 μg/ml doses of monoclonal antibody 163.31. However, in the presence of either UH or LMWH, all concentrations of monoclonal antibody 163.31 tested (in the presence of a constant amount of MAb 169.31) provided significant combinatorially effective inhibition, with essentially identical results obtained for the two heparin preparations. In the presence of heparin, the 1.25 μg/ml dose of 163.31 was more effective at inhibiting DNA synthesis than the 25 μg/ml dose of 163.31 in the absence of heparin. The presence of either UH or LMWH administered independent of any antibody had only a minimal effect on the mitogenic activity of serum.

Table 11

MAb (μg/ml)		CPM ± Std.Dev. (% Inhibition)		
163.31	169.31	(-) Heparin	(+) UH	(+) LMWH
0	0	31,002 ± 2,655	27,175 ± 1,518 (12%)	27,573 ± 947 (11%)
25	1	23,424 ± 371 (24%)	11,811 ± 365 (62%)	13,095 ± 409 (58%)
10	1	23,901 ±4,138 (23%)	12,317 ± 2,034 (60%)	11,829 ± 1,451 (62%)
5	1	29,366 ±1,652 (5%)	18,449 ± 1,802 (40%)	18,832 ± 1,530 (39%)
2.5	1	27,192 ± 2,351 (12%)	16,249 ± 4,075 (48%)	16,720 ± 2,674 (46%)
1.25	1	29,032 ± 1,012 (6%)	19,663 ± 1,455 (37%)	21,664 ± 1,485 (31%)

(see Example 18) and 10 μg/ml of heparin. The results, presented in Table 12, demonstrate that both the parent murine MAb 169.31 and the mouse/human chimeric anti-PDGFr-alpha antibody have similar inhibitory potency in the presence of either the parent murine anti-PDGFr-beta MAb 163.31 or the mouse/human chimeric anti-PDGFr-beta antibody.

TABLE 12

Antimitogenic Activities of Parent Murine and Mouse/Human Chimeric Anti-PDGFr-Alpha Antibodies Coordinately Administered With Heparin

Serum (2%)	Anti-PDGFr alpha (μg/ml)	Anti-PDGFr beta (10 μg/ml)	Heparin	CPM ± S.D.
+	Buffer	Buffer	No	7,242 ± 329
-	Buffer	Buffer	No	71 ± 6
+	169.31 (1.0)	163.31	Yes	2,278 ± 321
+	169.31 (0.1)	163.31	Yes	3,225 ± 366
+	Ch 169 (1.0)	163.31	Yes	2,879 ± 620
+	Ch 169 (0.1)	163.31	Yes	3,279 ± 985
+	169.31 (1.0)	Ch 163	Yes	2,715 ± 170
+	Ch 169 (1.0)	Ch 163	Yes	2,600 ± 575

Data are presented as counts per minute (cpm) ± standard deviation of [^{3}H]thymidine incorporated by baboon SMCs following stimulation with 2% baboon serum. Ch 169 = Mouse/human chimeric anti-PDGFr-alpha antibody, Ch 163 = Mouse/human chimeric anti-PDGFr-beta antibody.

antibodies were analyzed in independent administration assays and in coordinate administration assays using the antibody and heparin. The antibodies and heparin were added to appropriate test wells at 25 µl per well of a 40 X stock diluted in PBS. The antibody concentrations used are indicated in Table 13.

After administration of the test samples, the cells were incubated for 18 hours at 37°C. Mitogenic activity was assessed by uptake of [^{3}H] thymidine.

The results of the anti-PDGFr-beta MAb dose-response experiment, presented in Table 13, demonstrate that baboon serum-induced mitogenic activity was inhibited approximately 80% by a combination of 25 µg/ml 163.31, 1 µg/ml 169.31 and 2 Units/ml heparin. Similar results were obtained using the chimeric anti-PDGFr-beta MAb. Coordinate administration of each of the murine and chimeric anti-PDGFr-beta MAb, at 25 µg/ml, with the anti-PDGFr-alpha MAb 169.31, resulted in approximately 30% inhibition of baboon serum mitogenic activity. The anti-PDGFr-beta MAbs coordinately administered with heparin produced roughly 40% inhibition. Each of the antimitogenic agents, when administered independently, resulted in less than 30% inhibition of baboon serum mitogenic activity.

These results demonstrate that the mouse/human chimeric anti-PDGFr-beta antibody has similar inhibitory activity as that of the parent murine anti-PDGFr-beta antibody MAb 163.31. This activity provides a combinatorially effective inhibition of serum mitogenic activity on BVSMCs when the chimeric anti-PDGFr-beta antibody is administered coordinately with either the anti-PDGFr-alpha MAb 169.31, heparin, or a combination of anti-PDGFr-alpha MAb and heparin.

Table 13, continued

6.25	1	———	2,892 ± 382 (65%)
25	1	5,164 ± 519 (37%)	1,973 ± 374 (77%)

Data are presented as the counts per minute (cpm) ± standard deviation of [^{3}H]thymidine incorporated by baboon SMCs following stimulation with 2% baboon serum. Values of percent inhibition for both the parent and chimeric antibody studies were determined by comparing the cpm incorporated of [^{3}H]thymidine to a standard curve generated from the serum dose-response data presented below.

Serum Dose-Response

(%NBS)	(CPM+/-S.D.)
0	89 ± 0
0.125	444 ± 89
0.25	978 ± 62
0.5	2,190 ± 46
1	4,655 ± 248
2	7,768 ± 585

Example 19

Dose-Responsive Inhibition of Serum Mitogenic Activity on Baboon Vascular Smooth Muscle Cells by Heparin Administered Independently or Coordinately With Anti-PDGFr Antibodies

Baboon smooth muscle cells (BO54) were plated at a density of 2 x 10^4 cells/well in 24-well culture plates and grown for approximately 72 hours in DMEM containing 10% fetal calf serum at 37°C. The cells were then made quiescent by incubating them for 24 hours in Mito Media. The ability of heparin alone to inhibit mitogenic stimulation by 2% baboon serum was tested by adding a

Table 14

Dose-Responsive Inhibition of Serum Mitogenic Activity by Heparin Administered Independently or Coordinately With Anti-PDGFr Antibodies

			(+) MAb 163.31 (10 μg/ml) (+) MAb 169.31 (1 μg/ml)	
UH (U/ml)	cpm	Std.Dev.	cpm	Std.Dev.
0	4,694	586	3,532	276
0.06	5,816	495	2,662	377
0.18	4,653	368	2,030	246
0.55	4,900	527	1,133	157
1.67	4,473	405	714	56
5	4,451	357	526	43
15	3,628	255	369	16
LMWH (U/ml)	cpm	Std.Dev.	cpm	Std.Dev.
0	5,715	665	2,904	28
0.06	5,169	438	2,355	301
0.18	4,535	728	1,038	124
0.55	4,210	392	1,151	60
1.67	4,207	2422	641	9
5	3,642	734	678	52
15	3,457	369	380	35

Example 20

Inhibition of Smooth Muscle Cell Outmigration from Baboon Aortic Explants by Coordinate Administration of Anti-PDGFr MAbs and Heparin

Anti-PDGFr monoclonal antibodies were further tested in the presence or absence of heparin for their ability to decrease rates of baboon vascular smooth muscle cell outmigration from explants of baboon aortic tissue. Baboon aortic explants were set up essentially as described in Example 11. The explants were cultured in DMEM supplemented with insulin and transferin and containing the following test samples: 1) Anti-PDGFr-alpha MAb (169.31) and anti-PDGFr-beta MAb (163.31), each antibody at 25 μg/ml, 2) Unfractionated heparin (Sigma

in 24-well culture plates and grown for approximately 48 hours at 37°C in DMEM containing 10% fetal calf serum. The cells were incubated for 24 hours in Mito Media to become quiescent, then washed once with 4°C binding media (DMEM/Hams F-12, 25mM Hepes, 0.1%BSA). The binding activity of MAb 163.31 on PDGF-beta receptors was analyzed in the presence of heparin by adding four-fold dilutions of ^{125}I-labeled MAb 163.31 (^{125}I-163.31) in 4°C binding media (ranging from 2.1 x 10^6 to 1 x 10^3 cpm/ml) and 10 µg/ml heparin (Elkins-Sinn, Inc.) to the appropriate wells in triplicate in 1 ml aliquots. On a separate plate, the same dilution series of ^{125}I-163.31 was added without heparin. To determine the level of nonspecific binding by ^{125}I-163.31, a set of triplicate wells was set up on each plate containing 5.5 x 10^5 cpm/well of the ^{125}I-163.31 plus 25 µg/ml unlabeled 163.31. The plates were kept on ice while samples were being added, then incubated for 1.5 hour at 4°C on a rotary shaker. After washing 3x with PBS, the cells were incubated with an extraction buffer (PBS, 1% NP-40), and the extracts were harvested to 12 x 75 mm tubes and counted in a gamma counter. The results of the binding studies, presented in Table 16, demonstrate that the coordinate addition of heparin and antibody had no significant effect on antibody binding. Thus, the combinatorial effectiveness of coordinately administering heparin with the anti-PDGFr-beta antibodies, shown in the above examples, does not appear to be attributable to any stimulation by heparin of binding of the antibody to cell-surface PDGF-beta receptors.

each plate also contained 2.0 x 10^5 cpm/well of ^{125}I-PDGF-BB in addition to 1 µg/ml unlabeled PDGF-BB to determine the level of nonspecific binding by ^{125}I-PDGF-BB. The plates were kept on ice while samples were being added, and then incubated for 1.5 hour at 4°C on a rotary shaker. After washing 3x with PBS, the cells were incubated with an extraction buffer, and the extracts were harvested to 12 x 75 mm tubes and counted in a gamma counter.

The results, presented in Table 17, demonstrate that the presence of heparin had no significant effect on ^{125}I-PDGF-BB binding. Thus, the combinatorial effectiveness of coordinately administering heparin with the anti-PDGFr antibodies, shown in the above examples, does not appear to be attributable to any inhibition by heparin of the binding of PDGF-BB to cell-surface PDGF receptors.

Table 17

Saturation Binding Analysis of PDGF-BB on Baboon Vascular Smooth Muscle Cells in the Presence and Absence of Heparin

	Mean Specific cpm +/- Std.Dev.	
^{125}I PDGF-BB (applied cpm)	(+) Heparin	(-) Heparin
200,000	1,674.0 +/- 15.7	1,800.0 +/- 112.0
100,000	1,428.5 +/- 61.0	1,572.0 +/- 80.6
50,000	1,327.0 +/- 91.3	1,438.5 +/- 41.2
25,000	1,005.4 +/- 63.7	1,107.8 +/- 58.2

Table 18

Inhibition of ^{125}I-PDGF-BB binding to Smooth Muscle Cells By MAb 163.31 in the Presence and Absence of Heparin

	Mean Specific cpm ± S.D. (percent of control)	
MAb 163.31	(+) Heparin	(-) Heparin
24 µg/ml	888.0 ± 162.6 (39.5%)	715.0 ± 70.0 (36.6%)
8 µg/ml	911.0 ± 291.3 (40.5%)	944.0 ± 89.1 (48.3%)
2.7 µg/ml	1,176.0 ± 107.5 (52.3%)	1,021.0 ± 27.0 (52.2%)
0.9 µg/ml	1,221.0 ± 192.9 (54.3%)	1,136.0 ± 29.8 (58.1%)
0.3 µg/ml	1,549.0 ± 173.5 (68.9%)	1,354.0 ± 46.4 (69.3%)
0.1 µg/ml	1,682.0 ± 104.7 (74.8%)	1,687.0 ± 6.0 (86.3%)
0.033 µg/ml	2,174.0 ± 236.1 (96.7%)	1,726.0 ± 92.4 (88.3%)
0.0 µg/ml	2,248.0 ± 110.3 (100.0%)	1,955.0 ± 24.0 (100.0%)

Example 24

Continuous Intravenous Infusion of MAbs 169.31 and 163.31 into Baboons and Analysis of the Baboon Anti-Murine IgG Response

This study was designed to monitor circulating levels of the murine anti-PDGFr antibodies following continuous infusion by either intravenous or intraperitoneal routes. A pool of anti-PDGFr MAbs 163.31 and 169.31 was made with the two antibodies at approximate concentrations of 36 and 22 mg/ml, respectively. The

hours. The wells were washed with ELISA C buffer, then incubated with Reaction Buffer for approximately 1 minute. The reaction was stopped by the addition of 1N H_2SO_4, and the plates were read in an ELISA microtiter plate reader at 492 nm. Using the values obtained from the purified antibody samples, a standard curve was generated for each antibody, and the concentrations of the antibodies in the baboon plasma samples were determined from these curves.

The results, presented in Table 19, demonstrate that there was a peak in the circulating antibody levels at 1 day following pump placement in the i.v. infused animal, while the peak antibody levels were found at day 7 in the intraperitoneal infused animals. At days 14, 21 and 28 the circulating antibody levels were less than 1% of the peak levels measured at the earlier time points.

Table 19

Circulating Levels of Anti-PDGFr Antibody Following Continuous Infusion Into Baboons

	Animal A (IP)		Animal B (IP)		Animal C (IV)	
Day	MAb163	MAb 169	MAb163	MAb169	MAb163	MAb169
1	1000	1600	340	600	8000	6400
7	9000	7000	4000	3200	2500	200
14	14	64	5	6	8	40
21	8	50	5	8	4	18
28	7	40	1	2	3	14

Data are presented as ng/ml of circulating levels of the anti-PDGFr MAbs in baboon plasma at various times following the initiation of antibody infusion. IP: Intraperitoneal infusion, IV: Intravenous infusion.

Studies to measure potential baboon antibody generated against the infused murine antibodies were performed using ELISA. The results suggested that the low

vacutainer tubes at 0, 0.083, 0.25, 0.5, 1, 2, 4, 8, 12, 24, 72, 120, 168, 240, 336, 504, and 672 hours. Blood was spun, plasma was decanted, and radioactivity was determined in a gamma counter (80% efficiency). Antibody concentrations in the plasma were determined by comparing the counts in the blood to the initial specific activity of the radiolabeled antibody, taking into account the decay rate for ^{125}I. Analysis of the antibody concentrations in the plasma samples showed the half-life of the antibody to be approximately 50 hours.

Example 26
Development of a Sequential Arterial Injury Model in Baboons for Testing Antihyperplastic Agents and Treatments Following Vascular Injury

A model of sequential arterial injury was developed in the baboon to allow testing of the anti-PDGFr antibodies for their ability to inhibit experimentally induced intimal hyperplasia in primates. This model was designed to allow each animal to act as its own control by utilizing bilaterial arterial injuries introduced at 28 day intervals.

Baboons weighing approximately 10 kg each were used in this study. The initial surgical procedure closely resembled the vascular reconstructive procedure of balloon angioplasty used in clinical applications for treatment of human atherosclerosis. For each animal, an initial balloon denudation pull-back injury was made to the saphenous artery. On day 28 the animals underwent a second surgical procedure whereby the initially injured artery was excised, and the excised artery was perfusion-fixed *ex vivo* under 100 mm Hg pressure for 1 hour with a 10% formalin solution. Following excision of the first artery, the contralateral saphenous artery received a ballon denudation injury. Following the second 28-day

Table 20

Analysis of Intimal Lesion Development Following Sequential Balloon Denudation Arterial Injuries in the Baboon

Animal	Side	Intimal Area (mm^2)	Medial Area (mm^2)	I/M Ratio
Z6	R	0.1256	1.085	0.1151
Z6	L	0.0543	0.547	0.0995
Z8	R	0.1877	1.269	0.1493
Z8	L	0.1125	0.826	0.1344

Example 27

Evaluation of Anti-PDGF Receptor Antibody/Heparin Therapy to Inhibit Intimal Hyperplasia in Primates

Baboons weighing from 6 to 10 kg each were used to study the efficacy of anti-PDGFr MAbs and heparin. An initial balloon denudation pull-back injury was made to one saphenous artery of each animal using a 2F embolectomy catheter (Fogarty). At the time of injury femoral vein catheters and sub-cutaneous (SQ) osmotic pumps (Alzet) were inserted (twenty-eight day pumps, two pumps per animal). The pumps delivered a combined rate of 5 µl/hour into the femoral vein. During the first 28-day control period the pumps were loaded with placebo saline solution. During the 28-day period following the balloon injury, the animals received i.v. injections of placebo buffer on study days 1, 4, 8, 15, and 22.

On study day 29 a second surgical procedure was performed whereby the previously injured artery was excised and perfusion-fixed under 100 mm Hg pressure for 1 hour with a 10% formalin solution. The arteries were then divided into 10 sections of approximately 0.5 cm in length. Each tissue piece was embedded in paraffin, and

0.05% Tween-20 to eliminate non-specific binding. Dilutions of baboon serum, made in the same buffer, were added to the wells along with a dilution series of purified chimeric antibody diluted in control baboon serum. The plates were incubated at 37°C, then washed to remove unbound antibody. Goat anti-human IgG_4 antibody conjugated to horseradish peroxidase (Zymed, So. San Francisco, CA) was then added to the wells for one hour at 37°C. The wells were washed with PBS containing 0.05% Tween-20, then incubated with OPD substrate solution. The reaction was stopped by the addition of 1N H_2SO_4, and the plates were read in a Dynatech ELISA plate reader at 490 nm. Circulating antibody levels were determined by comparison of data points to a standard curve. Heparin levels were determined as described above. In addition, animals were monitored for any baboon antibody response directed towards the chimeric antibody by ELISA using a goat anti-monkey IgG conjugated with horseradish peroxidase (Cappel, Durham, NC).

Following the second 28-day period, the second injured artery was excised and perfusion-fixed *ex vivo* in a similar manner as the first artery.

A total of 15 animals were enrolled in the study. Preliminary studies, disclosed above, using the sequential injury model demonstrated that there was low variability between the arteries injured 28 days apart. Analysis of preliminary studies suggested that an n = 15 would be required to observe a 50% decrease in lesion development with a 95% confidence limit. The animals were divided into three groups of five for ease of surgical treatment. The side of the first procedure for each animal was randomized to eliminate any side-to-side variation.

Tissue sections were obtained from multiple blocks for each test artery as described above, and absolute intimal and medial areas were determined for each tissue section. The data for the multiple sections for

Table 21

Intimal Lesion Development, Control vs. Treatment

Summary of Mean Values

	Intimal Area (mm^2)		Medial Area (mm^2)		I/M Ratio	
	Control	Treated	Control	Treated	Control	Treated
Mean (n=12)	0.081	0.038	0.762	0.637	0.098	0.059
Std. Dev.	0.047	0.012	0.157	0.105	0.045	0.018
		53% decrease		16% decrease		40% decrease
		p=0.005		p=0.003		p=0.005

Statistical analysis was done using a paired Student t-test. A one-tailed test was done for the intimal area and the intimal/medial ratios, and a two-tailed test for the medial area.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be evident that certain changes and modifications may be practiced within the scope of the appended claims.

8. Use according to claim 7, wherein said antibody is a monoclonal anti-PDGF receptor antibody.

9. Use according to claim 7, wherein said antibody is an anti-PDGF-alpha receptor antibody.

10. Use according to claim 7, wherein said antibody is an anti-PDGF-beta receptor antibody.

11. Use according to claim 7, wherein said antibody is a humanized antibody.

12. Use according to claim 7, wherein said antibody is a single chain antibody.

13. Use according to claim 7, wherein said antibody is a chimeric antibody.

14. Use according to claim 13, wherein said antibody is a human-mouse chimeric antibody.

15. Use according to claim 14, wherein said chimeric antibody comprises mouse variable domains operably linked to human constant domains.

16. Products containing heparin and a PDGF antagonist as a combined preparation for simultaneous, separate or sequential use for the treatment of vascular hyperproliferative diseases.

17. Products according to claim 16, wherein said PDGF antagonist is a non-peptidic PDGF antagonist.

18. Products according to claim 16, wherein said PDGF antagonist is an anti-PDGF receptor antibody.

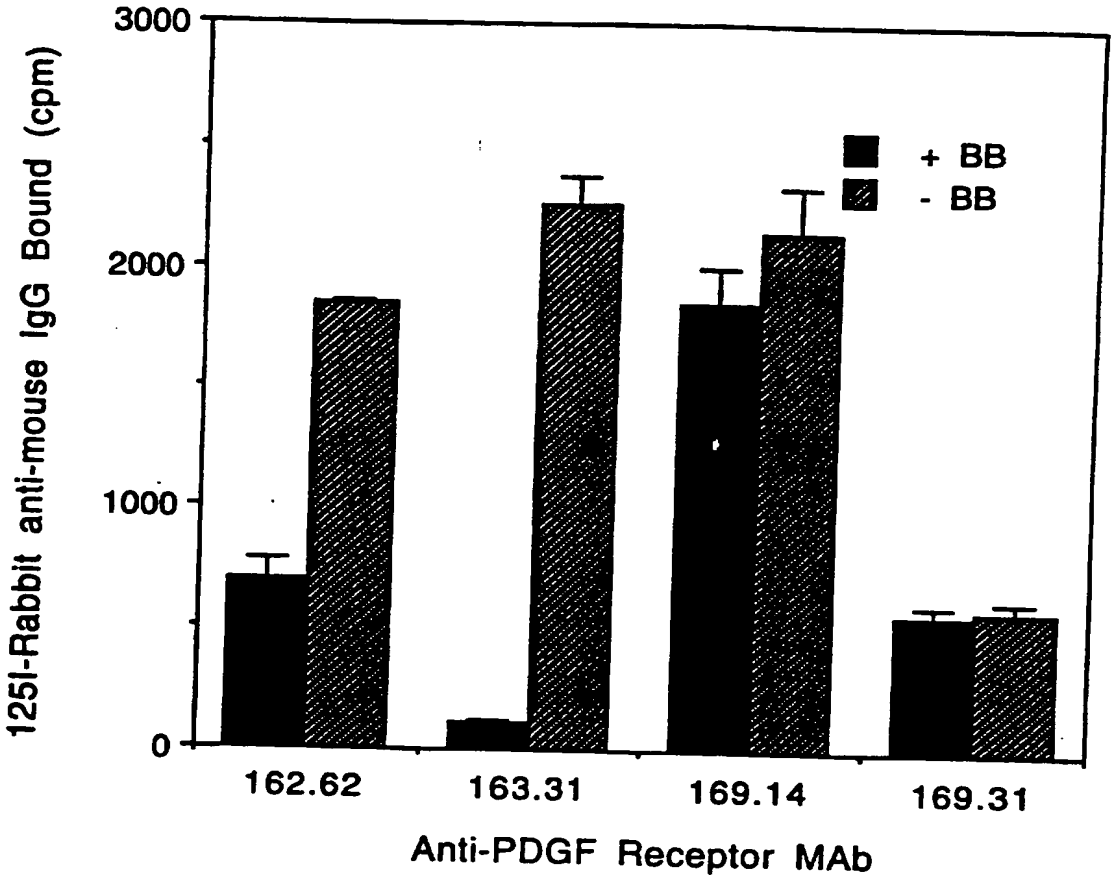

3000
2000
1000
0
125I-Rabbit anti-mouse IgG Bound (cpm)
+ BB
- BB
162.62
163.31
169.14
169.31
Anti-PDGF Receptor MAb

Figure 1

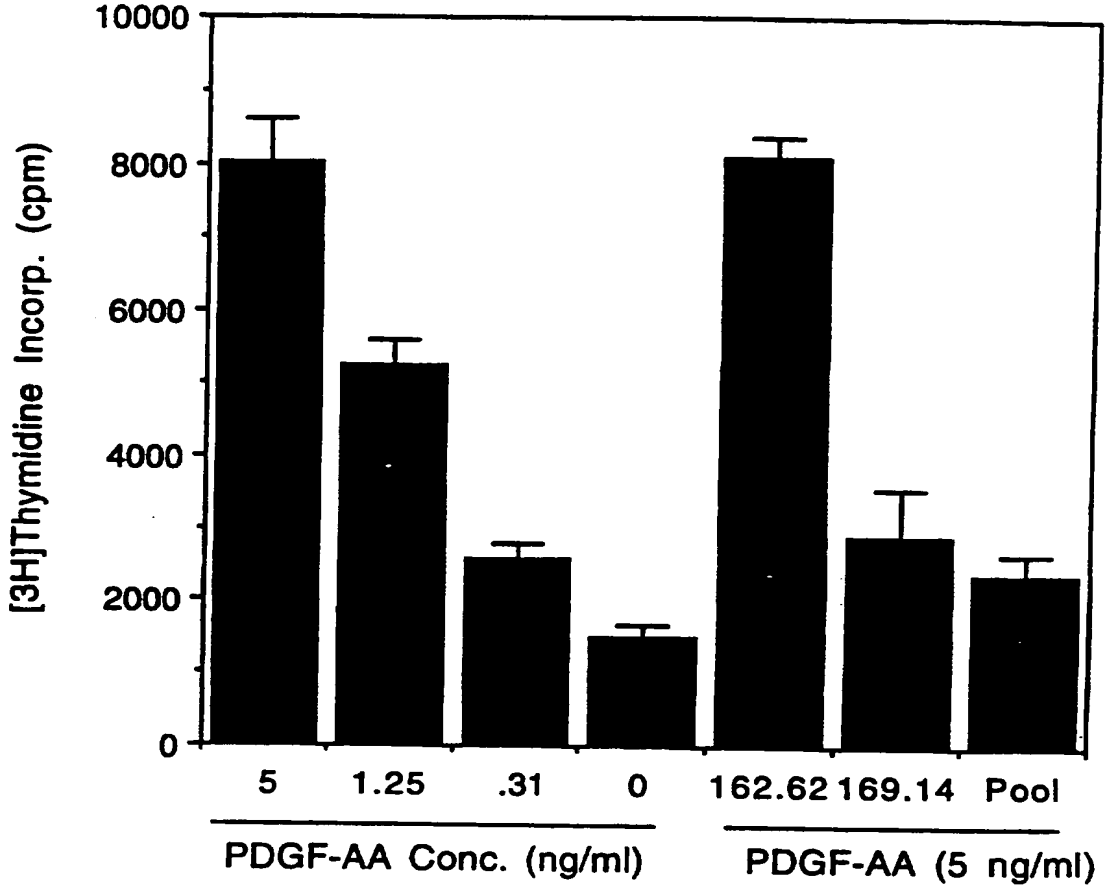
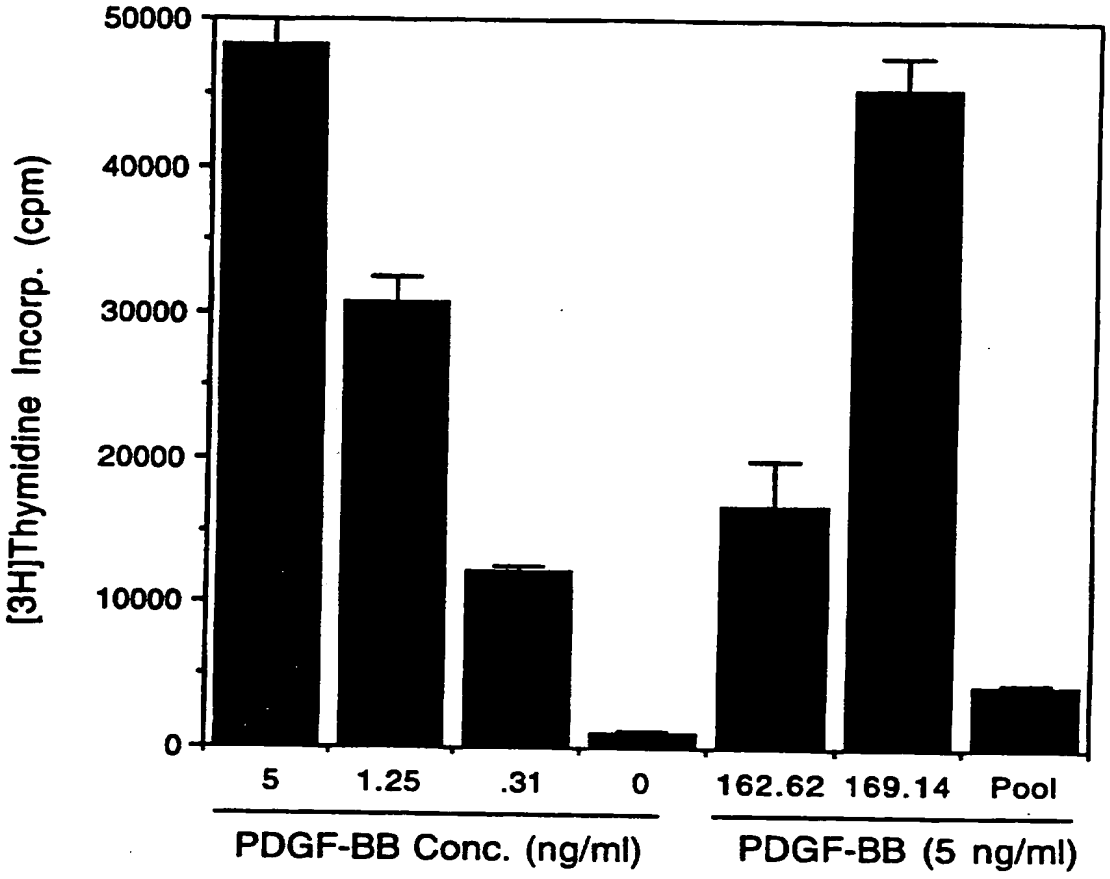

10000
8000
6000
4000
2000
0
[3H]Thymidine Incorp. (cpm)
5
1.25
.31
0
162.62
169.14
Pool
PDGF-AA Conc. (ng/ml)
PDGF-AA (5 ng/ml)

Figure 3A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40000
30000
20000
10000
0
[3H]Thymidine Incorp. (cpm)
5
1.25
.31
0
162.62
169.14
Pool
PDGF-BB Conc. (ng/ml)
PDGF-BB (5 ng/ml)

Figure 3C

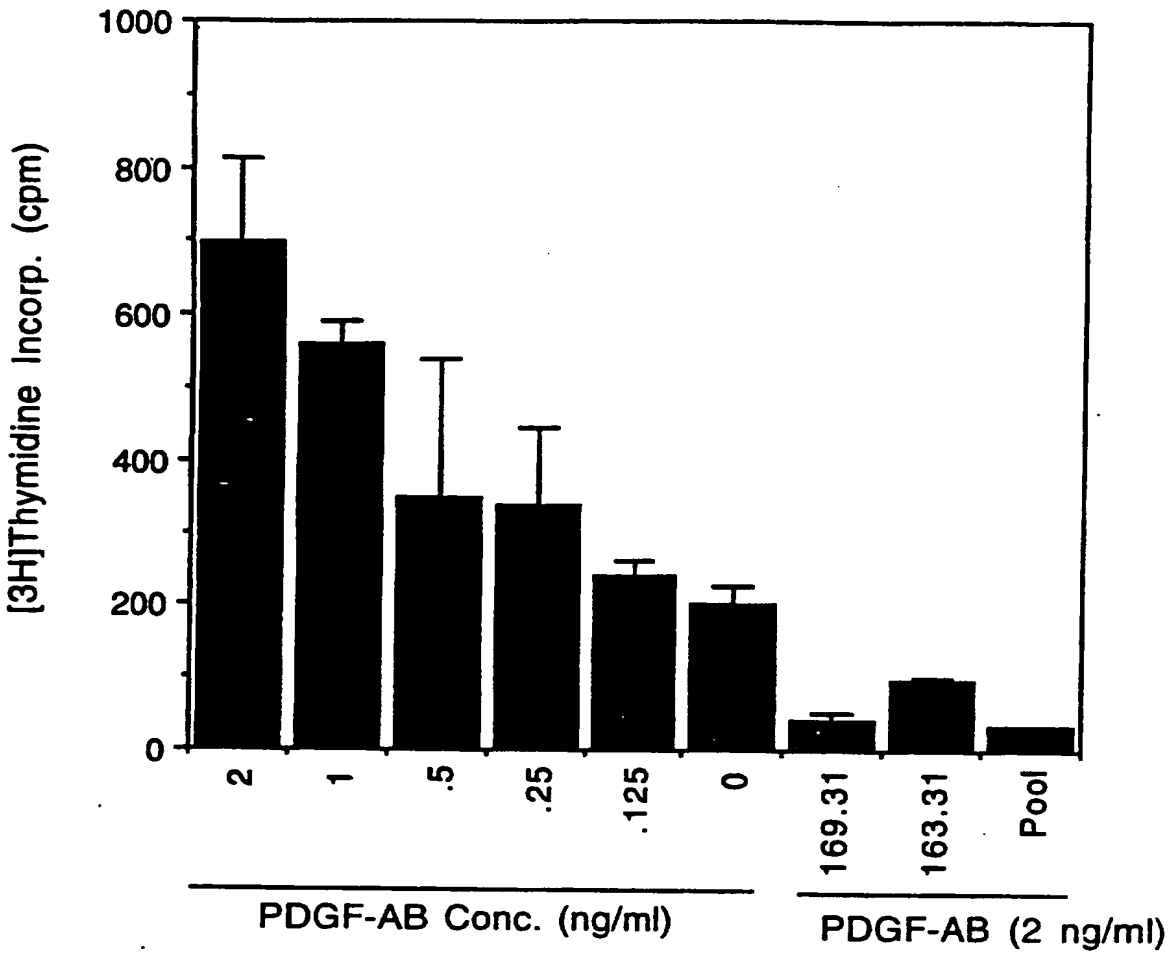
1000
800
600
400
200
0
[3H]Thymidine Incorp. (cpm)
2
1
.5
.25
.125
0
169.31
163.31
Pool
PDGF-AB Conc. (ng/ml)
PDGF-AB (2 ng/ml)

Figure 5

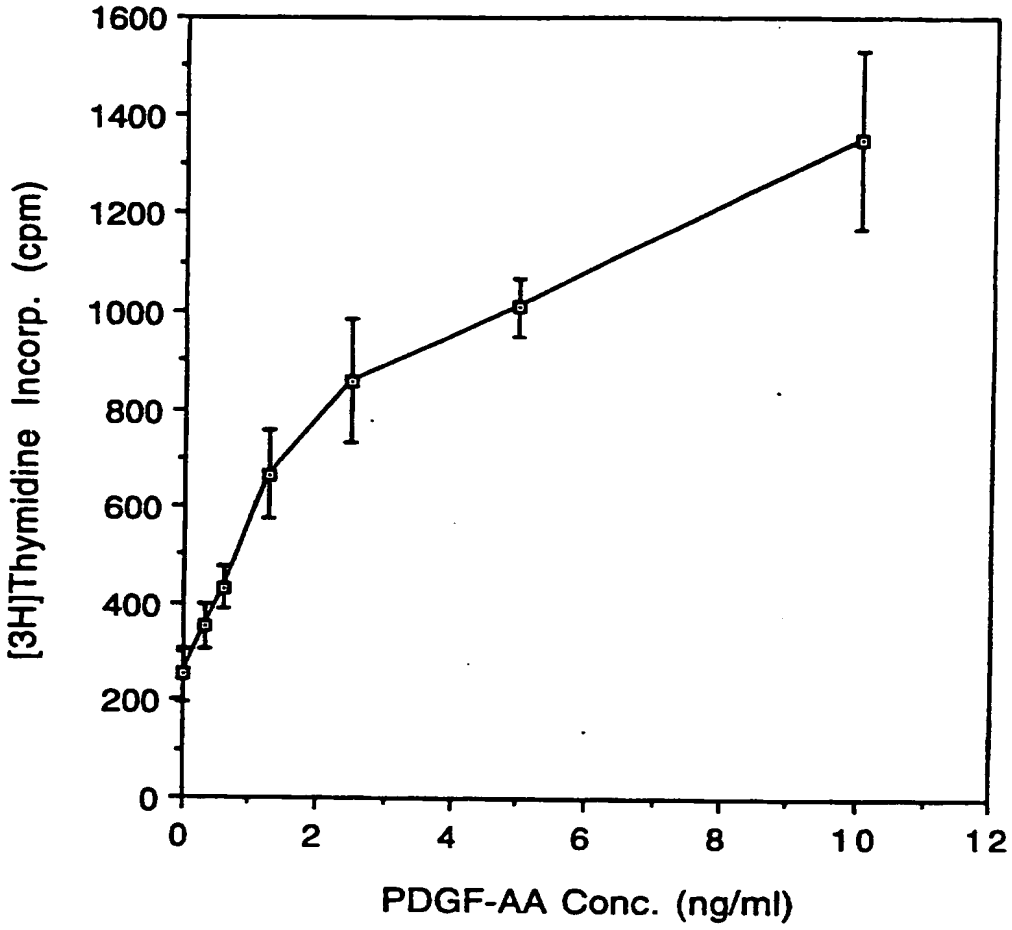
1600
1400
1200
1000
800
600
400
200
0
[3H]Thymidine Incorp. (cpm)
0
2
4
6
8
10
12
PDGF-AA Conc. (ng/ml)

Figure 7A

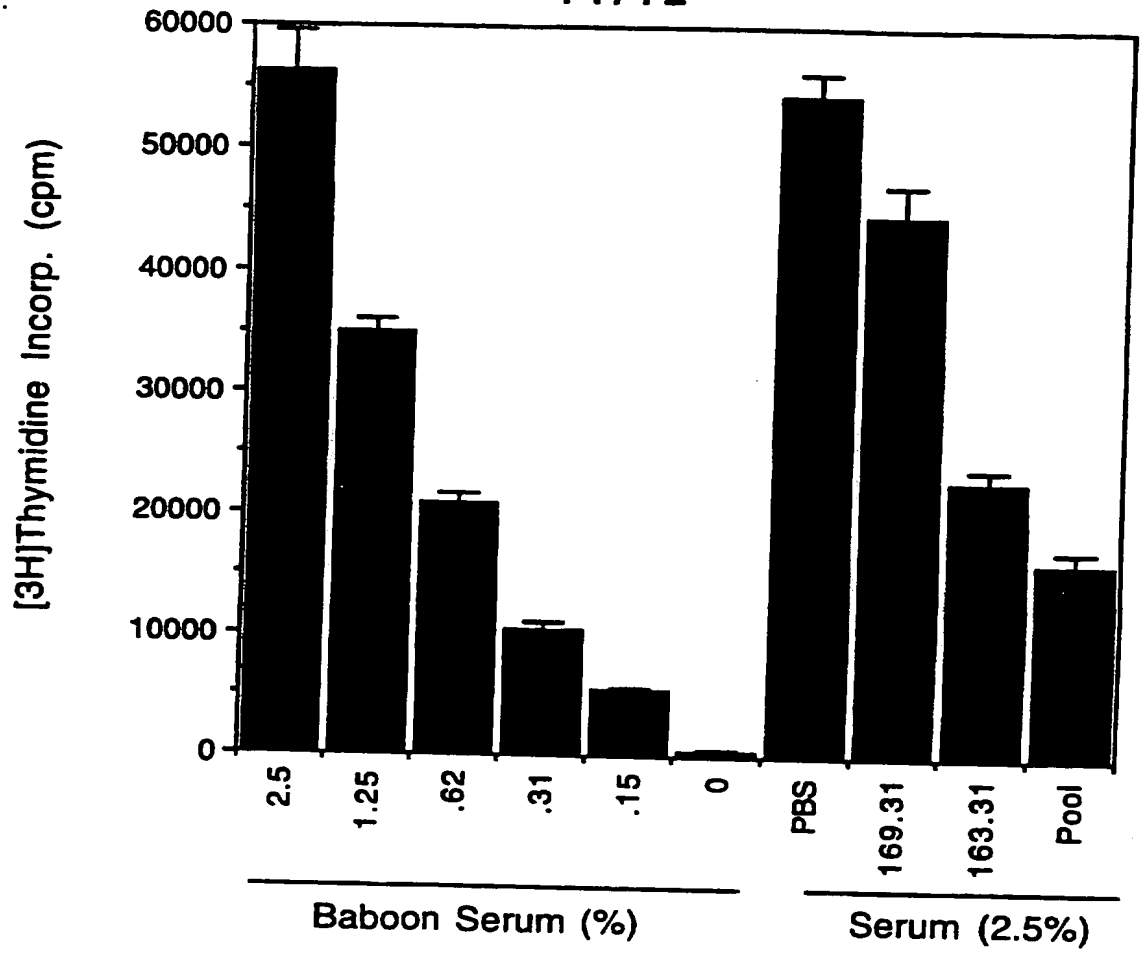
60000
50000
40000
30000
20000
10000
0
[3H]Thymidine Incorp. (cpm)
2.5
1.25
.62
.31
.15
0
PBS
169.31
163.31
Pool
Baboon Serum (%)
Serum (2.5%)

Figure 8

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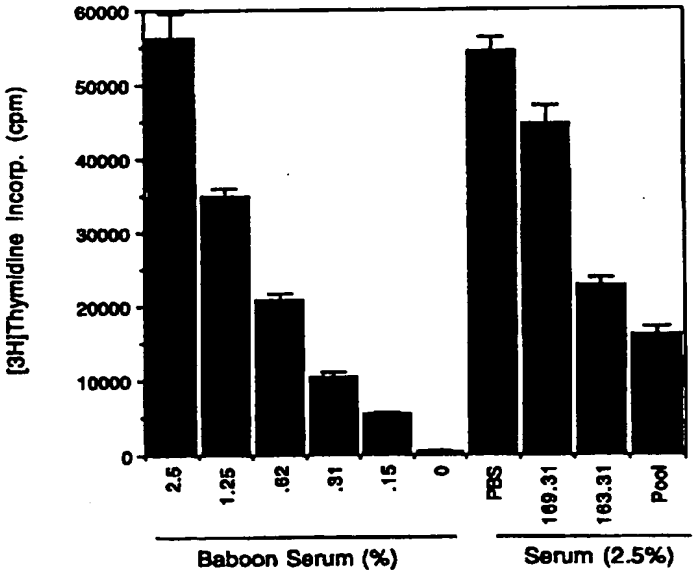

(57) Abstract

Methods for inhibiting intimal hyperplasia in the vasculature of mammals, including primates, are disclosed. The methods comprise coordinately administering to the mammal a PDGF antagonist and heparin. The antagonist can be a non-peptidic antagonist or an anti-PDGF receptor antibody, such as an anti-PDGF-alpha receptor antibody or an anti-PDGF-beta receptor antibody. The methods are useful in reducing intimal hyperplasia due to, for example, vascular injuries resulting from angioplasty, endarterectomy, reduction atherectomy or anastomosis of a vascular graft.

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 95/16683

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K39/395 A61K31/715 A61K31/365

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,94 19016 (ZYMOGENETICS INC ;UNIV WASHINGTON (US)) 1 September 1994 see the whole document	1-25

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
24 June 1996	05.07.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Leherte, C

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INTERNATIONAL SEARCH REPORT

nformation on patent family members

Inter nal Application No
PCT/US 95/16683

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9419016	01-09-94	CA-A- 2156825	01-09-94
		EP-A- 0686044	13-12-95